

Thesis for the degree of doctor of philosophy (phd)

**DESIGNING AND SYNTHESIS OF PEPTIDES FOR
SELECTIVE BINDING OF TOXIC METAL IONS AND
STUDYING THEIR METAL COMPLEXES**

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I. INTRODUCTION AND THE AIM OF THE WORK

Diseases and abnormalities caused by heavy metal ion poisoning are common nowadays. The lead(II) ion triggers disorders of nerval- and neurodevelopment, it incorporates into the bonds and it is able to damage the central nervous system. The cadmium(II)ion is responsible for the itai-itai disease, induces the fragility of the bones and provokes kidney failure. *The Bioinorganic Research Group of Inorganic and Analytical Department at University of Debrecen* have studied the coordination chemistry of lead(II)- and cadmium(II) ions for a long time. The investigations, which covered the amino acids and peptides as well, confirmed that the main binding site for the metal ions is the sulphur donor atom of the thiolate group; however, high stability complexes can be formed with the oxygen donor atom containing carboxylate group. Furthermore, based on the previous studies, it became apparent that the number, position and type of the donor atom play an important role in the metal binding ability of the ligands, but these ligands do not show significant selectivity toward, for example, the vital zinc(II)ions. Similar conclusion can be drawn from the results of the nickel(II) containing systems, but in comparison with the above mentioned metal ions, the essential difference is that the nickel(II)ion can induce the deprotonation of amide group resulting in various coordination types. Changing the amino acids of peptides there is an effective way to investigate the competition of the ligands for the metal ions. Thus, it was an important aspect in the design of sequences that the corresponding peptide should be able to bind only the toxic metal ions selectively in the human organism.

The aim of our work was to find peptides which may be able to bind the toxic lead(II) and cadmium(II) ions selectively. We aimed for the

solid phase synthesis of the three types of peptides and the characterization of the lead(II) and cadmium(II)-complexes, complete with the study of the zinc(II) and nickel(II)-complexes of some of the ligands. We sought the answers to the following questions:

- 1) how does the stability of lead(II)-complexes depend on the increase in the number of the carboxylate group in peptides containing different numbers of aspartic acid?
- 2) do the N-terminally protected small peptides containing one or two cysteinyl residue show any selectivity in the binding of lead(II) and cadmium(II)?
- 3) how does the complexation with lead(II) ion depends on the distance of the cysteinyl residues and on the other donors of the residues?
- 4) how does the coordination behaviour and metal ion binding ability depend on the distance between the N-termini amino group and the N-termini cysteinyl residue, in the case of lead(II) and nickel(II) complexes of N-terminally free, two cysteinyl containing oligopeptides?

II. EXPERIMENTAL METHODS

One part of the investigated peptides (**Figure 1**) was synthesized by **solid phase peptide synthesis** using Liberty 1TM (CEM, Matthews, NC) automatic microwave-assisted peptide synthesizer in the Bioinorganic Chemical Research Group. Acylated peptides at N-terminus and peptides containing free amino groups were synthesized using the Fmoc/tBu technique. Amidated terminal at C-terminus was provided by Rink Amide AM type resin. In order to activate the carboxyl and amide groups, the TBTU/HOBt/DIEA strategy was used. N,N-dimethylformamide (DMF) was used as a solvent for the synthesis. The rest of the peptides were purchased commercially (**Figure 2**). The purity of the lyophilized product was checked using **high pressure liquid chromatography** (RP-HPLC) technique using analytical Teknokroma Europa Peptide C18 (250 mm x 4,6 mm, pore size: 120 Å, particle size: 5 µm) type analytical column and Jasco MD-2010 plus diode array detector. The detection was carried out at 222 nm, which is characteristic of the maximum absorption wavelength of peptide bond. Gradient elution was used during the investigations. Water, containing 0.1 V/V% TFA and acetonitrile was used as eluent, where the ratio of the acetonitrile increased to 20 V/V% within 0-15 minutes, then it decreased to 0 V/V% within 15-20 minutes. The flow rate was 0.8-1.0 ml/min depending on the type of the peptide.

pH potentiometric titration was used to determine the peptide content of the studied ligands. This method was also used to determine the protonation constants of the peptides and the stability constants of the metal ion complexes. Applying the experimental data PSEQUAD and SUPERQUAD computational program was used to carry out the calculations. The measurements were carried out in part

on a Mettler Toledo T50 instrument ($I=0,20 \text{ mol/dm}^3 \text{ KNO}_3$ ion strength, $T=298,0 \text{ K}$) and in part by using Molspin titration equipment ($I=0,20 \text{ mol/dm}^3 \text{ KNO}_3$ or KCl , $T=298,0 \text{ K}$). The measurements were carried out in aqueous media beside different metal ion-ligand ratio. Based on the parameters of the defined system the concentration distribution curves were plotted with MEDUSA program.

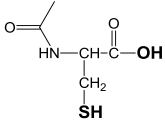
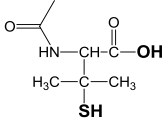
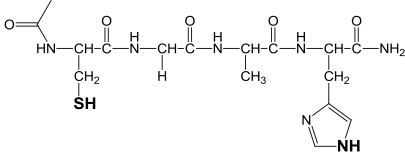
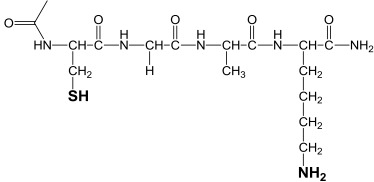
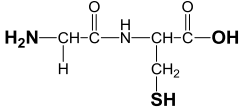
The **UV-Vis spectrophotometric measurements** were carried out within the wavelength range of 200-900 nm (in the case of Ni(II)ion containing systems) and 200-400 nm (for the Pb(II)- and Cd(II)-ion containing systems) using 1,000 cm quartz cuvette by a Perkin Elmer Lambda 25 dual-beam photometer. The spectra were recorded at different metal ion to ligand ratios, at different pH values.

In the case of the Ni(II)ion containing systems **circular dichroism (CD) spectroscopy** was also used. The CD spectra were recorded on a Jasco J-810 type spectrometer available at the Department of Organic Chemistry at the University of Debrecen in 220-800 nm using 1,000 cm and 0,100 cm cuvettes. The systems were measured at different metal ion-ligand ratio at different pH values.

In order to trace of the complex formation and to check the purity of the peptides, **NMR spectroscopic** measurements were carried out on a Bruker Avance DRX 400 MHz FT-NMR instrument. For the evaluation of the spectra TopSpin 3.5 and MestReNova 9.0 softwares were used.

Electrospray ionization mass spectrometry (ESI-MS) was used to identify the synthesized ligands and the stoichiometry of the metal-complexes in some cases. The examinations were fulfilled using maXis II MicroTOF-Q type Qq-TOF MS with ESI ionization source in negative and positive method.

<p style="text-align: center;">SH</p>	Ac-AAAC-NH ₂
<p style="text-align: center;">SH</p>	Ac-SAAC-NH ₂
<p style="text-align: center;">SH</p> <p style="text-align: center;">OH</p>	Ac-CGAD-NH ₂
<p style="text-align: center;">SH</p> <p style="text-align: center;">SH</p>	Ac-SCCS-NH ₂
<p style="text-align: center;">SH</p> <p style="text-align: center;">SH</p>	Ac-CSC-NH ₂
<p style="text-align: center;">SH</p> <p style="text-align: center;">SH</p>	Ac-CGSC-NH ₂
<p style="text-align: center;">SH</p> <p style="text-align: center;">SH</p>	Ac-CSSC-NH ₂
<p style="text-align: center;">SH</p> <p style="text-align: center;">SH</p>	Ac-CSSACS-NH ₂

	Ac-Cys
	Ac-Pen
	Ac-CGAH-NH ₂
	Ac-CGAK-NH ₂
	GlyCys
<p>Figure 2: Structural formulae of the commercially available peptides.</p>	

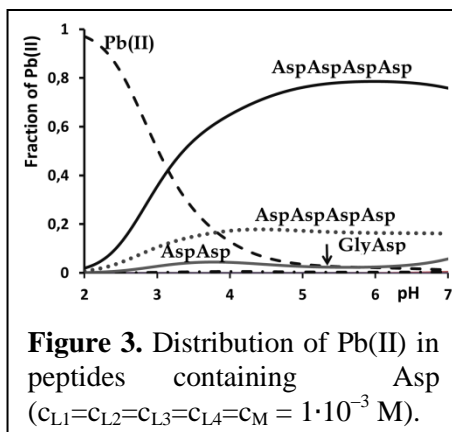
III. NEW SCIENTIFIC ACHIEVEMENTS

3.1 Method validation was carried out for photometric study of systems containing Pb(II).

- Lead(II)-trifluoroacetate solution, which does not have UV-absorption in the studied wavelength range, was prepared.
- The absorption maximum related to the number of the coordinated thiolate groups around the lead(II) ion was determined. The characteristic absorption maximum of one coordinated thiolate group is between 276-277 nm while in the case of two thiolate groups it is between 309-311 nm wavelengths.

3.2. The Pb(II) complexes of peptides containing different number of aspartic acid were characterized.

- We found that the effect of the groups of β -carboxylate is obvious, they bonded directly to the metal ion, increasing the stability of the complexes.
- In the case of the four peptides (GlyAsp, AspAsp, AspAspAsp and AspAspAspAsp) only mononuclear complexes appear with composition 1:1, while



bis(ligand) complexes were not detected even in the case of solutions containing high ligand excess.

- The stability of the complexes increases with the increasing number of the β -carboxylate groups (**Figure 3**) but the size of the ligand or its higher negative charge does not influence it.
- The carboxylate donor groups behave as anchor groups and the terminal amino group remains protonated in the protonated species.
- Comparing the stability of these peptide complexes to those of the previous studied metal ions the stability order is the following:

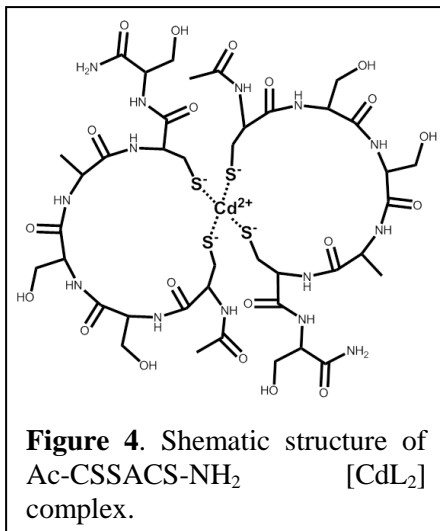


3.3. Pb(II) complexes of N-terminally protected peptides containing one or two cysteins were characterized.

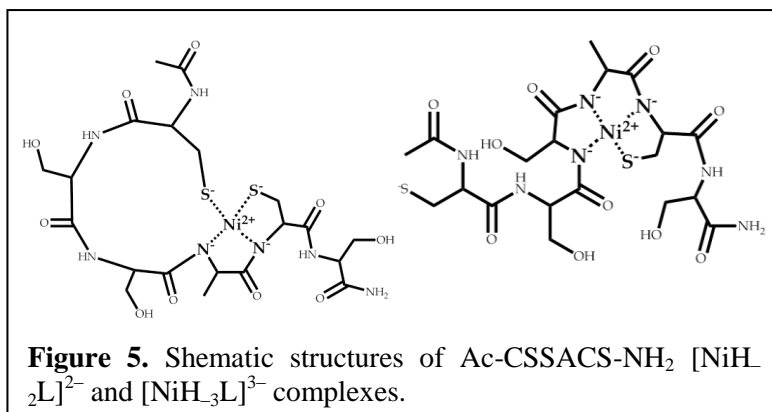
- It was found that in the case of the lead(II) complexes of peptides containing one cystein only, the thiolate group takes part in the metal binding and the effect of other amino acid side chains (lysine or hystidine) is negligible.
- Bis(ligand) complexes are also formed in some cases, and based on the spectrophotometric data, the formation of the tris(ligand) complexes was also supposed.
- In equimolar solutions the formation of polymer complexes was confirmed by spectrophotometric measurements.
- If there are two cysteins in the peptide, the thiolate groups of both amino acids are involved in the complex formation.
- Bis(ligand) complexes are formed only in the case of Ac-SCCS-NH₂ ligand. However, the formation of polymer structured complexes is also assumed.
- It was confirmed that the number and position of the cystein residues in the peptide influence the stability of the metal complexes. The peptides containing two cysteins have much higher lead(II) ion binding ability than the peptides containing one cystein or peptides containing cystein and another donor group.

3.4. Cd(II)-, Zn(II)- and Ni(II) complexes of some N-terminally protected peptides containing one or two cysteins were studied

- It was found that the Ac-SAAC-NH₂, the Ac-AAAC-NH₂, and the Ac-CSSACS-NH₂ peptides form bis- or tris(ligand) complexes with Cd(II)- and Zn(II) ions in all cases (**Figure 4.**) but in the presence of Ni(II) ion only mono complexes are formed.



- For the Ni(II) ion, too, the thiolate group of the cysteine is the anchor group, but in this case the Ni(II) promotes the deprotonation of peptide nitrogens thus (S⁻,N⁻,N⁻,N⁻) coordinated species exists above ~11,0 pH.
- In the Ac-CSSACS-NH₂ [NiH₂L]²⁻ complex the ligand is coordinated via (S⁻,N⁻,N⁻,S⁻) donor groups and the amide nitrogen can substitute one thiolate sulphur atom only in [NiH₃L]³⁻ complex formed at higher pH (**Figure 5.**).



- Based on the calculated pM data of the examined systems ($c_L = 1,0 \cdot 10^{-5}$ M, $c_M = 1,0 \cdot 10^{-6}$ M), it was proven that the metal binding ability of the protected Ac-CSSACS-NH₂ peptide containing two cysteins is much higher for all four studied metal ions than that of peptides containing one cystein.

3.5. Pb(II)-, Cd(II)- and Zn(II) complexes of N-terminally free peptides containing two cysteins were studied, the difference between the coordination mode of complexes of various peptides and different metals were determined.

- It was concluded that the lead(II) ion forms high stability complexes with all three oligopeptides (CSSACS-NH₂, ACCSSACS-NH₂ and SSCSSACS-NH₂).

- It was stated that the stoichiometry of the complexes is similar but they differ in coordination modes.

- The stability of [PbL] complex of CSSACS-NH₂ peptide is higher than that of the other two peptide [PbL] complexes due to the stable tridentate coordination of the ligand. (**Figure 6**).

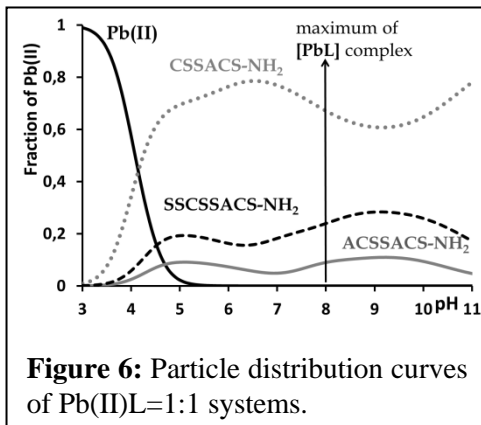
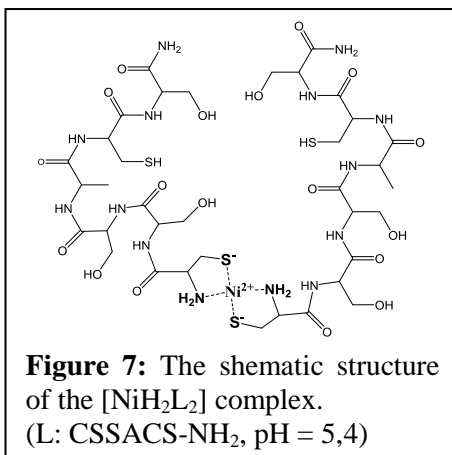


Figure 6: Particle distribution curves of Pb(II)L=1:1 systems.

- It was found that the (S⁻,S⁻) donor set is the main binding site for the lead(II) ion if the -CXXXC- sequence is further from the N-terminal part of the molecule. The participation of the N-terminal amino group in the coordination is significantly reduced or negligible.
- The formation of the bis(ligand) complexes was proven in the case of ACSSACS-NH₂ and SSCSSACS-NH₂ peptides.
- The main difference between these bis(ligand) complexes was found to be that the two ligands coordinated to the Pb(II)-ions via 3S⁻ donor atoms while in the case of Cd(II) and Zn(II) the CdH_xL₂ and ZnH_xL₂ complexes the 2x(S⁻,S⁻) (x = 0-2) coordination mode is characteristic due to the different geometry.

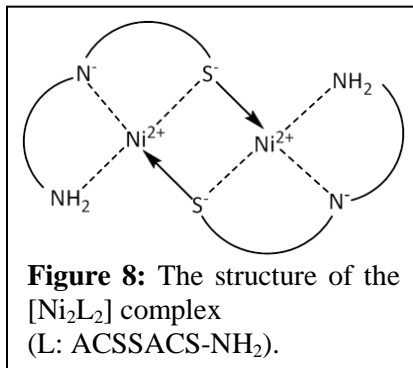
3.6. Ni(II) complexes of N-terminally free peptides containing two cysteinyl residues were characterised

- In the case of Ni(II)-CSSACS-NH₂ system, the formation of precipitate occurred above pH 6.5 therefore only the stability constant for the [NiH₂L₂] (Figure 7.) complex could be determined by pH-potentiometry, in which the coordination mode is 2X(NH₂,S⁻).



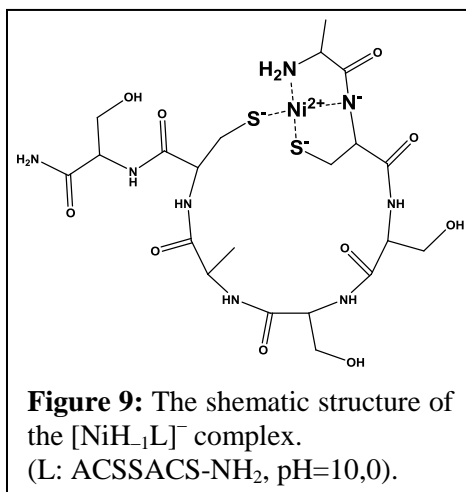
- It has been stated that parallel to the deprotonation of the second cysteinyl thiol group, complexes with polymeric structure are formed. It has been proven, that under strongly basic conditions, the C-terminal cysteinyl residue is the main binding site for Ni(II), resulting in the formation of complexes with (S⁻,N⁻,N⁻,S⁻) or (N⁻,N⁻,N⁻,S⁻) coordination mode.
- It has been stated that in the case of ACSSACS-NH₂ ligand the N-terminal part of the peptide is the main binding site for the Ni(II) ion, thus the metal ion is coordinated by (NH₂,N⁻,S⁻) donor set.

- We have shown that in the case of ligand excess, bis(ligand) complex is formed, in which the second ligand is able to saturate the coordination sphere of the metal ion via the sulphur donor atom of thiolate group. In equimolar solution, two (NH₂,N⁻,S⁻)



coordinated nickel(II) units join together via thiolate bridge resulting in the formation of dimeric complexes (**Figure 8**).

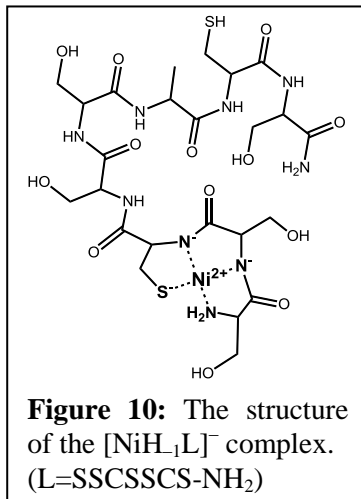
- We have stated that in basic pH range the C-terminal thiolate group saturates the coordination sphere of the Ni(II) ion parallel to its deprotonation, breaking the bis complex formed in the presence of ligand excess as well as the dimeric complexes formed in equimolar solution (**Figure 9**).



- We have confirmed that if the thiolate group occupies the third position from the N-termini, the N-terminal part of the peptide will also be the main binding site for the Ni(II) and the coordination sphere of the Ni(II) is saturated by (NH₂,N⁻,N⁻,S⁻)

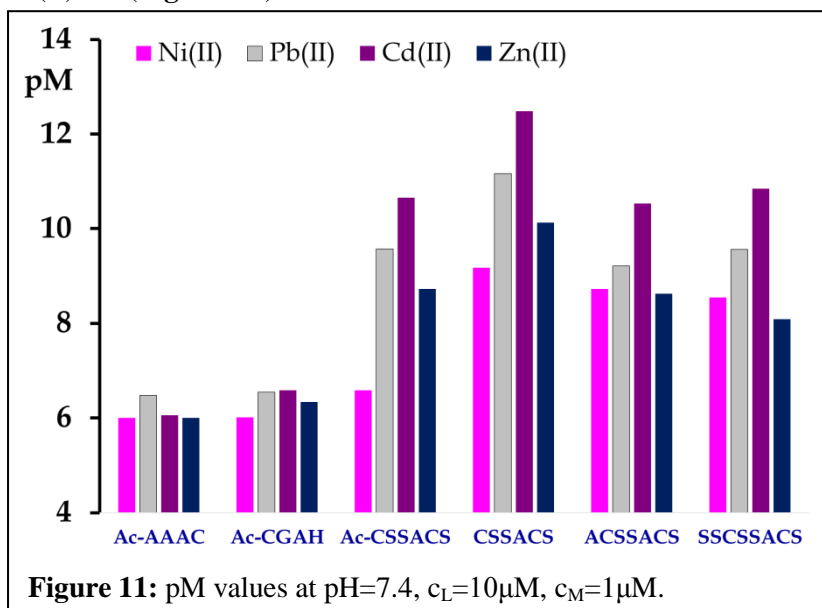
donor set, therefore the mononuclear $[\text{NiH}_1\text{L}]^-$ complex is the main species over a wide pH range (**Figure 10**).

- In the case of ACSSACS-NH₂ and SSCSSACS-NH₂ peptides, both of the N- and C-terminal thiolate groups are binding sites for the Ni(II), therefore the formation of dinuclear complexes was detected in the presence of metal ion excess.



- In equimolar solution of the octapeptide, a significant amount of dinuclear species was also formed above pH 9.

Taking all of the aforementioned results into consideration, we have stated that the thiolate group of the cysteine is a preferred binding site for the metal ions. Participation of the thiolate sulphur donor atom in the metal ion coordination results in the formation of lead(II) complexes with higher stability than that of the β -carboxylate group of the aspartic acid. The N-terminally protected small peptides are not able to selectively bind the investigated metal ions, because of the formation of poorly soluble complexes. Although high selectivity could not be observed for the N-terminally free two cysteinyl containing peptides either, we have stated that these ligands are able to bind the toxic Pd(II)- and Cd(II) ions with higher stability than the essential Zn(II) ion (**Figure 11.**).



IV. POSSIBLE APPLICATIONS OF THE RESULTS

Based on the previous results, it can be seen that the versatility of the ligands for metal ion binding is wide. From a biological point of view, the most important group of these ligands are the peptides, with different amino acid content. These peptides are able to form high stability complexes with essential, as well as toxic metal ions, therefore these are not metal ion selective agents.

The research work done during my PhD is a basic research on the field of bioinorganic and coordination chemistry, but the results clearly showed that the metal ion binding ability of the peptides can be changed by the modification of the amino acid sequence and the donor atoms.

Based on these results, the possible continuation of this research could be the design and synthesis of those peptides, in which further side chain donors can be found beside the two cysteinyl residues, making the fine tuning of the metal ion binding ability possible.

These new peptides could be promising candidates for medical treatments, for the selective removing of lead(II) and cadmium(II) ions from the human body.

V. PUBLICATIONS

Articles related to the dissertation

1. Györgyi Szunyog, Katalin Várnagy

Lead(II) complexes of oligopeptides containing two cysteine residues

Inorganica Chimica Acta, 472 (2018) 157-164.

IF: 2,433

2. Norbert Lihi, Márton Lukács, Mária Raics, Györgyi Szunyog, Katalin Várnagy, Csilla Kállay

The effect of carboxylate groups on the complexation of metal ion with oligopeptides – Potentiometric investigation

Inorganica Chimica Acta, 472 (2018) 165-173.

IF: 2,433

3. Györgyi Szunyog, Alíz Laskai, Dóra Szűcs, Imre Sóvágó, Katalin Várnagy

A comparative study on the nickel binding ability of peptides containing separate cysteinyl residues

Dalton Transactions (submitted, under revision)



Registry number: DEENK/317/2019.PL
Subject: PhD Publikációs Lista

Candidate: Györgyi Szunyog
Neptun ID: G7JOL0
Doctoral School: Doctoral School of Chemistry
MTMT ID: 10056733

List of publications related to the dissertation

Foreign language scientific articles in international journals (2)

1. **Szunyog, G.**, Várnagy, K.: Lead(II) complexes of oligopeptides containing two cysteine residues.
Inorg. Chim. Acta. 472, 157-164, 2018. ISSN: 0020-1693.
DOI: <https://doi.org/10.1016/j.ica.2017.07.067>
IF: 2.433
2. Lihí, N., Lukács, M., Hadháziné Raics, M., **Szunyog, G.**, Várnagy, K., Kállay, C.: The effect of carboxylate groups on the complexation of metal ion with oligopeptides - Potentiometric investigation.
Inorg. Chim. Acta. 472, 165-173, 2018. ISSN: 0020-1693.
DOI: <http://dx.doi.org/10.1016/j.ica.2017.07.032>
IF: 2.433





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List of other publications

Foreign language abstracts (1)

3. **Szunyog, G.**, Várnagy, K.: Lead Complexes of Cysteine Containing Peptides.

In: 13th European Biological Inorganic Chemistry Conference : Program & Book of Abstracts,
Hungarian Chemical Society, Budapest, 160, 2016. ISBN: 9789639970670

Total IF of journals (all publications): 4,866

Total IF of journals (publications related to the dissertation): 4,866

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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